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Confocal Microscopy of the Skin in the Diagnosis of X-Linked Alport Syndrome

To the Editor:

Alport syndrome is an inherited disorder of type IV collagen, the major collagenous constituent of basement membranes (Timpl and Brown, 1996). The hallmark of the disease is persistent microscopic hematuria, often associated with proteinuria, progressive renal failure, ocular abnormalities, and high-tone sensorineural hearing loss (Kashtan, 1998). The majority (about 85%) of Alport kindred show X-linked dominant inheritance, which is caused by mutations in the COL4A5 gene located in the Xq22 region that encodes for the α5(IV) chain of type IV collagen (Mochizuki *et al*, 1994; Renieri *et al*, 1996). Mutations of the COL4A5 gene result in the loss of α5(IV) chain in basement membranes (Yoshioka *et al*, 1994); in general, absence of α5(IV) expression in a male patient, or a mosaic distribution in a female, is diagnostic of X-linked Alport syndrome (Grunfeld, 2000).

α5(IV) is present in normal glomerular basement membrane (GBM) and also in the normal epidermal basement membrane (EBM) (Peissel *et al*, 1995); its absence results in typical ultrastructural lesions of the GBM, virtually diagnostic of the disease, whereas no morphologic abnormalities have been observed in the EBM (Kashtan *et al*, 1986; Nakanishi *et al*, 1994).

Immunohistochemical study of the skin, however, is at present considered the procedure of choice in evaluating a patient with microhematuria who is suspected of having X-linked Alport syndrome, being much less invasive than renal biopsy. Absence of α5(IV) in the EBM, observed in nearly 80% of hemizygotes, or a segmental pattern in heterozygotes, is diagnostic of the syndrome (Kashtan, 1998; Kashtan, 1999; Pirson, 1999; Grunfeld, 2000).

Unfortunately, in most published series a number of patients exist in whom COL4A5 mutations are associated with a positive, linear staining (Kashtan and Michael, 1996; Kashtan, 1998; 1999).

Immunohistochemical demonstration of α5(IV) expression is currently achieved by the standard indirect immunofluorescence method, a simple and highly specific technique. Yet, standard fluorescence microscopy has a somewhat low resolving power, depending upon the use of relatively thick (4–6 μm) tissue sections.

The aim of this study was to verify whether the use of alternative microscopy techniques for immunofluorescence could prevent the occurrence of "false negative" results. This was achieved by confocal laser scanning microscopy (CLSM), which produces high-resolution images of thick biologic samples (Paddock, 1999).

We selected from a total of 45 probands with proven X-linked Alport syndrome those cases in which routine immunofluorescence for α5(IV) revealed a positive, linear, and continuous staining pattern. Eight symptomatic patients were studied (four males and four females); clinical and molecular data are summarized in **Table I**. The diagnosis was based on the identification of a mutation on the COL4A5 gene and/or on the presence of the typical ultrastructural lesions of the GBM in renal biopsies performed on the patient or on an affected relative. Two patients (#1 and #4) had a negative family history, but showed a COL4A5 mutation and a typical ultrastructural appearance of the GBM. In patient #2 a COL4A5 mutation was not identified but a typical family history was present (her child displayed a negative α5(IV) staining along the GBM). All male patients but one (#8) had proteinuria; conversely, proteinuria was present only in one female patient (#3). Renal function was normal in all patients. Mild neurisensorial deafness and a lens opacity was present in patient #6. Our work was performed with full approval of our institution and all experiments were conducted in compliance with the Helsinki Principles for laboratory work with human tissue.

Punch (3 mm) skin biopsies were performed at the axillary fold in six patients, and in three patients skin tissue was obtained during percutaneous renal biopsy. Mean age at biopsy was 23.2 y (range 6–45 y). Informed consent was obtained from all patients and/or their parents.

Unfixed skin samples were embedded in OCT, snap frozen in liquid-nitrogen-cooled isopentane, and stored at –80°C until used. Ten samples of normal skin were obtained as controls at surgery from patients with malignant tumors (squamous cell carcinoma or melanoma). Immunohistochemistry was performed using monoclonal antibodies against α1(IV) and α5(IV) chains (Weislab, Lund, Sweden). Sections of all patients and controls were independently examined by two observers with a Nikon microscope equipped with epifluorescence illumination optics. The same sections were subsequently examined with a Sarastro 2000 confocal laser scanning microscope by a third investigator, without knowledge of the specimen category (patients *versus* controls).

Results of conventional fluorescence microscopy confirmed in all cases of Alport syndrome the presence of a linear, uninterrupted signal for α5(IV). Confocal microscopy, performed on the same specimens, revealed that the pattern of distribution of α5(IV) was indeed abnormal, with the three-dimensional reconstruction of the EBM appearing as a discontinuous sheet. The disrupted pattern varied from focal interruptions (i.e., small to medium sized areas of negative staining, mostly linear in shape, irregularly distributed along the whole length of the examined EBM) to a coarsely granular distribution of the fluorescent dye (i.e., large, irregularly shaped areas of negative staining surrounded by rims or spots of positive staining) (see **Table II** and

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Reprint requests to: Andrea Onetti Muda, M.D., Dipartimento di Medicina Sperimentale e Patologia, Università La Sapienza, Viale Regina Elena, 324 (Policlinico) I-00161 Roma, Italy; Email: andrea.onettimuda@uniroma1.it

Abbreviations: CLSM, confocal laser scanning microscopy; EBM, epidermal basement membrane; GBM, glomerular basement membrane.

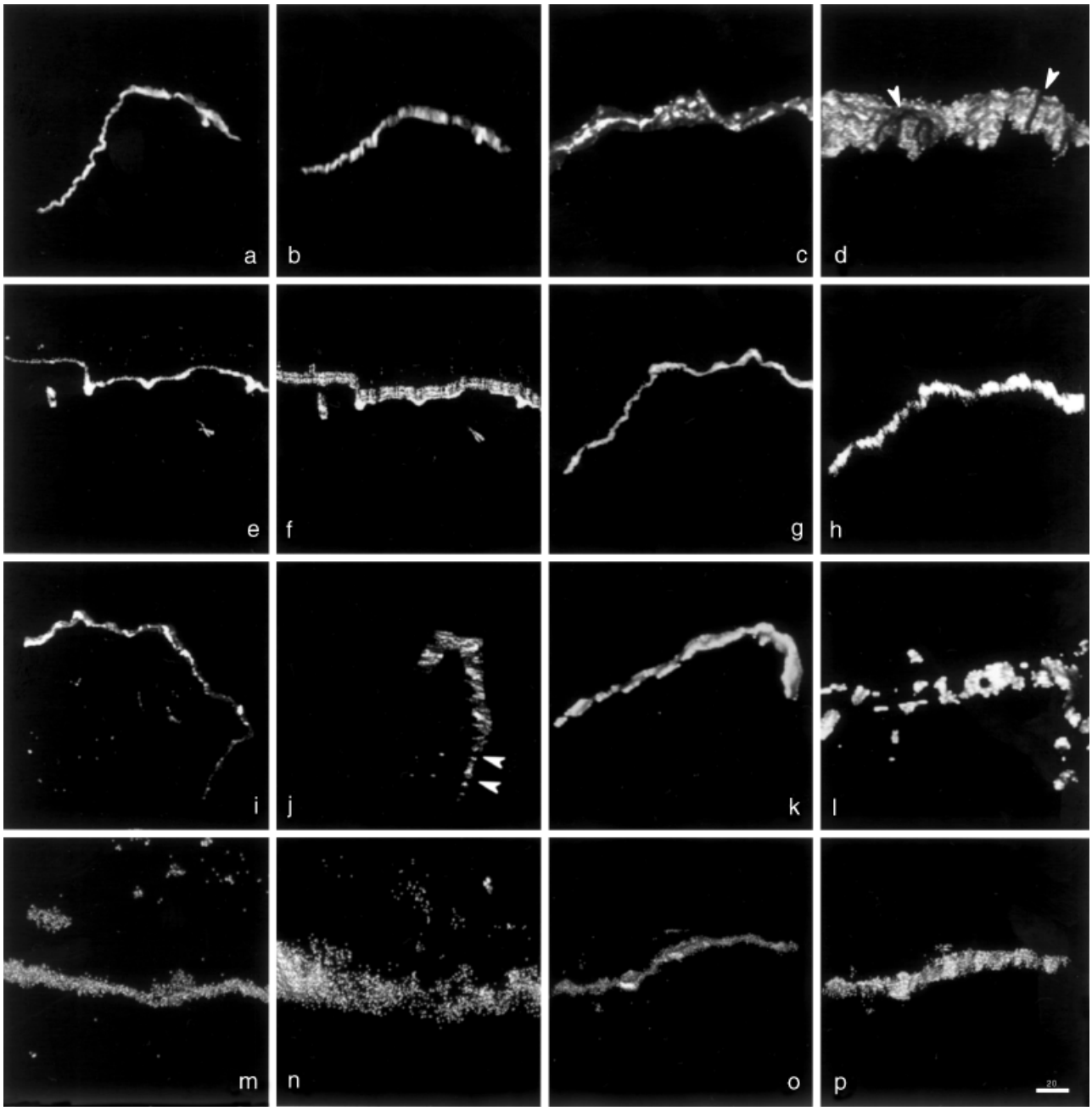

Figure 1. Immunohistochemical localization of α5(IV) chain in the skin of Alport cases. Thick (15–20 μm) cryostat sections of frozen tissues were fixed in acetone and stained by the indirect method; primary antibodies were diluted 1:50 (α1(IV)) or 1:5 (α5(IV)) in phosphate-buffered saline with 0.5% bovine serum albumin. Incubation was carried out for 1 h at room temperature, followed by 30 min incubation with 1:20 solution of fluorescein isothiocyanate conjugated secondary antibody (Dako, Glostrup, Denmark). Sections for α5(IV) immunostaining were denatured in 6 M urea 0.1 glycine HCl buffer, pH 3.5, 30 min at room temperature, before the immune reaction. Optical sectioning was performed in three different areas of each specimen (two at the extremities of the skin fragment and the third in the middle portion) using a 60 × oil immersion objective; vertical and horizontal scans were made at 0.5 μm intervals and 30 images were reconstructed through ImageSpace software (Molecular Dynamics). (*a*), (*b*) case #1; (*c*), (*d*) case #2; (*e*), (*f*) case #3; (*g*), (*h*) case #4; (*i*), (*j*) case #5; (*k*), (*l*) case #6; (*m*), (*n*) case #7; (*o*), (*p*) case #8. In each case, the reconstructed image is observed in front view (*left*) or has been partially rotated, showing the specimen from the top (*right*). Focal interruptions are best appreciated in the partially rotated images (see also **Table II** for detailed description). *Bar*: 20 μm.

Fig 1 for details of each case). CLSM reconstruction of skin samples from control cases, in contrast, always exhibited a homogeneous, uninterrupted pattern of distribution (**Fig 2**). As expected, immunohistochemical distribution of α1(IV) chain along the EBM was not different in Alport cases and in controls (not shown).

Our study shows that the apparently normal skin distribution of α5(IV) chain in a number of X-linked Alport patients is the consequence of an optical artifact. Such false negative cases can be the consequence of "minor" mutations affecting the COL4A5 gene (Naito *et al*, 1996; Jais *et al*, 2000), or the result of random inactivation of the X chromosome, in which a high proportion of cells carry a normal, activated chromosome (Guo *et al*, 1995; Belmont, 1996). Whatever mechanism is at work, the result is a segmental distribution pattern, with smaller areas without signal surrounded by a positive staining. Such distribution is easily overlooked by conventional fluorescence microscopy.

Table I. Clinical and molecular data

Patient	Sex	Age at biopsy (y)	Family history	Microscopic hematuria	Hearing loss	Ocular lesions	Mutation Name	Exon	Reference
1	F	20	−	+	−	−	p.P456.P458del	21	Renieri *et al*, 1996
2	F	45	+	+	−	−	None		
3	F	36	+	+	−	−	p.G1214X	41	
4	F	21	−	+	−	−	c.4363_4364ins4	45	Massella *et al*, 1994
5	M	13	+	+	−	−	p.G325R	17	
6	M	23	+	+	+	+	c.IVS14 + 2T > C	4 (IVS)	
7	M	25	+	+	−	−	c.IVS14 + 2T > C	4 (IVS)	
8	M	6	+	+	−	−	p.P212del	11	

Table II. α5(IV) chain distribution along the EBM

Patient	Standard fluorescence microscopy	Confocal microscopy and three-dimensional reconstruction
1	Diffuse, homogeneous	Focal interruptions, small size, mostly linear, along the EBM
2	Diffuse, homogeneous	Focal interruptions, small size, irregular shape, along the EBM
3	Diffuse, homogeneous	Innumerable interruptions, very small size, irregular shape, along the EBM
4	Diffuse, homogeneous	Focal interruptions, small to medium size, mostly linear, along the EBM
5	Diffuse, with slight reduction of intensity at downward projections of rete ridges	Several interruptions, small to medium size, irregular shape, along the EBM
6	Diffuse, with slight reduction of intensity at downward projections of rete ridges	Coarsely granular signal, with large interruptions, along the EBM
7	Diffuse, homogeneous	Several interruptions, mainly of large size, irregular shape, along the EBM
8	Diffuse, homogeneous	Focal interruptions, small size, irregular shape, along the EBM

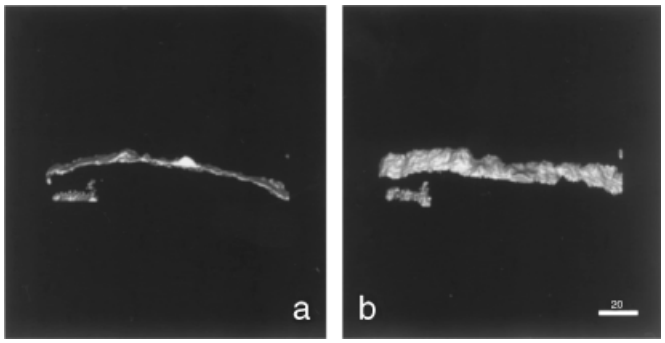

Figure 2. Normal human skin. Cryostat section immunostained for α5(IV) chain; indirect immunofluorescence using fluorescein isothiocyanate conjugated secondary antibody. (*a*), (*b*) Three-dimensional reconstruction of 30 consecutive optical sections; the reconstructed image is observed in front view (*a*) or has been partially rotated, showing the specimen from the top (*b*). Signal distribution along the dermo-epidermal junction is homogeneous, with no interruptions. *Bar*: 20 μm.

Use of confocal microscopy allowed us to demonstrate that the apparent linearity of the fluorescent signal along the EBM was the result of an artifact; in a two-dimensional view, a negative spot that is positioned along the optical path of a positive signal will be prevented from being visible, conferring on the specimen an overall appearance of continuity. Three-dimensional reconstruction of the EBM, followed by rotation along its axis, made observation from different angles possible, thus revealing the hidden features of the region of interest. The sensitivity of immunofluorescence study of skin biopsies is greatly improved, and the number of false negative results is virtually eliminated.

An intriguing point of our study is the finding of a segmental distribution pattern in affected male patients, and a convincing explanation is not available. Faint staining for α5(IV) chain has been described in male patients with COL4A5 mutations, however (Nakanishi *et al*, 1994; Naito *et al*, 1996), and the possibility exists that the use of a more accurate detection system like CLSM may reveal small areas of improper formation/incorporation of the triple helix.

The approach currently recommended to confirm a suspected diagnosis of Alport syndrome calls for the study of α5(IV) skin expression, followed, if not diagnostic, by kidney biopsy (Kashtan, 1999). Our data indicate that CLSM examination of the same tissue fragment could reveal a segmental diagnostic pattern, therefore postponing, or even preventing, a more invasive diagnostic procedure, and should be considered before kidney biopsy.

Andrea Onetti Muda, Laura Massella,* Konstantinos Giannakakis, Alessandra Renieri,† Gianfranco Rizzoni,* and Tullio Faraggiana
Dipartimento di Medicina Sperimentale e Patologia, Università La Sapienza, Roma; *Ospedale Bambino Gesù – IRCCS, Roma; †Genetica Medica, Università di Siena, Siena, Italy

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High Seroprevalence of Anti-*Mycoplasma Fermentans* Antibodies in Patients with Malignant Aphthosis

To the Editor:

Mycoplasmas are wall-less bacteria causing infections of mucosal tissue. Many mycoplasmas are commensals, others, such as *Mycoplasma (M) fermentans*, are associated with rheumatoid arthritis and other joint disorders (Schaeverbeke *et al*, 1996). Most, if not all, mycoplasmas contain macrophage activating components. Studies on a macrophage activator from mycoplasmas, comparable in activity with the lipopolysaccharide of gram-negative bacteria, led to the identification of macrophage-activating lipopeptide (MALP)-2 from *M. fermentans*. MALP-2 is a small lipopeptide originating from MALP-404, a 40 kDa large lipoprotein (Calcutt *et al*, 1999). MALP-2 and other mycoplasmal lipoproteins have an unusual N-terminus. It consists of a cystein bearing a dihydroxypropyl group in thioether linkage. Both hydroxy groups are esterified with long chain fatty acids, whereas the amino group is free. This distinguishes mycoplasmal lipoproteins from those in walled bacteria. Mycoplasmal lipoproteins are exceptionally good antigens, because they have a built-in adjuvant site in the form of the lipid-substituted N-terminus. Thus, MALP-404 is an immunodominant determinant in *M. fermentans*. MALP-404 is expressed by most *M. fermentans* strains, including those isolated from the joints of patients with reactive arthritis (Mühlradt, unpublished).

Like other bacterial proteins, MALP-404 contains the peptide motif-G------F, which can be presented by HLA-B51 (Falk *et al*, 1995). This transplantation antigen is associated with malignant aphthosis (Sakane *et al*, 1999). Malignant aphthosis (Adamantiades–Behçet's disease) is a chronic, multisystemic inflammatory disorder, which is clinically characterized by relapsing oral aphthous and genital ulcers, ocular, and vascular lesions. The disease may affect small and large vessels in almost all organs. Malignant aphthosis is a universal rare disorder with varying prevalence, occurring endemically in the Eastern Mediterranean area and in Central and East Asia, with a peak onset in the third decade of life (Zouboulis, 1999). A microbial infection has been implicated in the development of the disease to explain the strong inflammatory reactions observed (Zouboulis and May, in press), the activation of monocytes and macrophages, and the induction of proinflammatory cytokines and chemokines detected (Zouboulis *et al*, 2000; Alpsoy *et al*, 2003). Recently, several reports demonstrated that crude fractions of lipoproteins derived from different mycoplasma strains showed macrophage-stimulatory activities by inducing the production of proinflammatory cytokines. For all these reasons, we investigated the presence of antibodies against MALP-404 in the sera of patients with malignant aphthosis and examined a possible correlation of *M. fermentans* infection with the disease in an ethic committee-approved case–control study.

The 22 patients [(10 female, 12 male; median age 37 y; originating from Germany (n = 3), Turkey (n = 12), and other countries (n = 7)] and 14 gender-, age-, and land of origin-matched healthy controls [(seven female, seven male; median age 36 y; originating from Germany (n = 4), Turkey (n = 7), and other countries (n = 3)] were recruited after providing written consent. Patients fulfilled the criteria of the International Study Group for Behçet's Disease (1990). The presence of the MALP-404 antigen was detected in the serum of the subjects examined by the western blot method. Demographic and background characteristics were displayed using summary statistics. The primary and secondary variables were evaluated using χ^2 tests. For all comparisons, a significance level of 0.05 was applied.

From the 22 patients with malignant aphthosis, seven (32%) were MALP-404-positive; among them three patients (14%) with

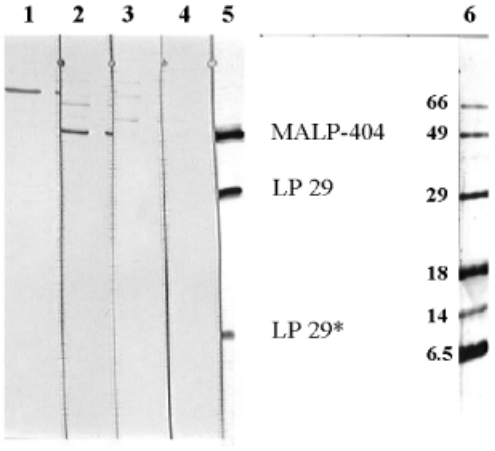

Figure 1. Western blot of selected sera from patients with malignant aphthosis (Adamantiades–Behçet's disease). Mycoplasma lipoprotein antigens were enriched by cold octyl glucoside extraction of a *M. fermentans* strain isolated from the knee joint of a rheumatoid arthritis patient. The antigen mixture was separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted to a PVDF membrane. *Lanes 1 to 4*: sera from patients with malignant aphthosis; *lane 5*: monoclonal antibodies against the indicated lipoproteins (positive control); *lane 6*: molecular weight markers. Note typical positive reaction to MALP-404 in *lane 2*, and strong reaction with an unidentified *M. fermentans* antigen in *lane 1*.

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Address correspondence and reprint requests to: Prof. Dr. Christos C. Zouboulis, Department of Dermatology, University Medical Center Benjamin Franklin, The Free University of Berlin, Fabeckstrasse 60-62, 14195 Berlin, Germany. Email: zouboulis@medizin.fu-berlin.de

Abbreviations: M, mycoplasma; MALP, macrophage-activating lipopeptide.